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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR §1.53(c).

INVENTOR(S)		
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[] Additional inventors are being named on the 0 separately numbered sheets attached hereto.

TITLE OF THE INVENTION (280 characters max)

Reagents that Bind the Amyloid Form of the AB Peptide

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Respectfully submitted,

Signature

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PROVISIONAL APPLICATION FOR PATENT

under

37 CFR §1.53(c)

TITLE: REAGENTS THAT BIND THE AMYLOID FORM OF THE AB PEPTIDE

APPLICANT: PAUL TAYLOR MARTIN

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REAGENTS THAT BIND THE AMYLOID FORM OF THE AB PEPTIDE

Background

[0001] This invention provides polypeptides, nucleic acids encoding the polypeptides, and methods of using the polypeptides or nucleic acids to diagnose and/or treat diseases associated with plaque formation in brain tissue, such as Alzheimer's Disease (AD). For example, the polypeptides of the invention can specifically target the amyloid form of the Ab1-40 peptide in plaques of Alzheimer's patients. The peptides can comprise 20 amino acid residues intramolecularly cross-linked by two cysteines. They have been shown to bind to AD plaques in vitro and can be conjugated with compounds that could label or treat the plaques. This method is an improvement over existing methods that use either antibodies, large proteins or small molecules such as dyes. Antibodies or large proteins generally fail to cross the blood-brain barrier and small molecules or dyes are generally unable to specifically recognize the plaques. The polypeptides, nucleic acids encoding the polypeptides, and methods of using the polypeptides or nucleic acids can be used to identify, diagnose and/or treat disorders associated with plaque formation in brain tissue.

Brief description of the drawings

[0002] Figure 1 shows staining of amyloid $A\beta_{1-40}$ by phage peptides. Both phage peptide sequences selected for $A\beta_{1-40}$ amyloid binding stained amyloid deposits *in vitro*. Final clones 2 and 4 are the DWGKGGRWRLWPGASGKTEA sequence. This sequence identified both small and large (0.5-5μm) accumulations of $A\beta_{1-40}$ amyloid. Final clone 6 is the PGRSPFTGKKLFNQEFSQDQ sequence. This sequence stained $A\beta_{1-40}$ amyloid aggregates more poorly, but still stained well above background levels. None of the ten starting clones randomly picked (Starting clone 6 is shown) stained when used at the same concentration. Bar is 5 μm.

[0003] Figure 2 shows immunublotting of monomeric $A\beta_{1-40}$ by phage peptides. Monomeric $A\beta_{1-40}$ was separated on a 4-12% Bis/Tris gradient gel and blotted with either anti-Aß antibody or with phage clones. No starting or final phage peptide clones recognized monomeric $A\beta_{1-40}$ peptide. Blotting with a monoclonal antibody that recognizes $A\beta_{1-40}$ is shown as a control for protein transfer.

[0004] Figure 3 shows recombinant Aß-binding peptide binds with high affinity to $A\beta_{1-40}$ amyloid *in vitro*. A recombinant cysteine-linked form of the DWGKGGRWRLWPGASGKTEA sequence was produced as a fusion protein with thioredoxin in *E. coli* (Thio-Aß). Recombinant Thio-Aß was purified and

binding to $A\beta_{1-40}$ amyloid was measured. Recombinant Thio-Aß bound $A\beta_{1-40}$ amyloid with a Kd of 60 nM. Binding was saturating by 200nM. Recombinant purified thioredoxin (Thio) showed no binding at any of the concentrations used. Errors are SEM for n=6.

[0005] Figure 4 shows specific binding of Thio-Aß to amyloid plaques in Alzheimer's disease brain. Recombinant Aß-binding peptide conjugated to thioredoxin (Thio-Aß) was used to stain brain samples from normal subjects and those with Alzheimer's disease (AD). Thio-Aß did not stain any structures in normal brain tissue, but heavily stained amyloid plaques from AD brains. Background staining was allowed to increase to allow visualization of cells within the section, but this staining was not caused by Thio-Aß. Thioredoxin (Thio) did not stain amyloid plaques in AD brains when added at the same concentrations. Bar is 100µm for panels on the left and 25 µm for panels on the right.

[0006] Figure 5 shows binding of synthetic peptides to $A\beta_{1-40}$ amyloid *in vitro*. Two biotin-labeled peptides, Biotin-DWGKGGRWRLWPGASGKTEA and Biotin-AECDWGKGGRWRLWPGASGKTEACGP, were tested for binding to $A\beta_{1-40}$ amyloid. The peptide containing flanking cysteines bound with a Kd of 320 nM, while the peptide lacking these cysteines did not bind with significant affinity below 5µM. Errors are SEM for n=6.

[0007] Figure 6 shows specific staining amyloid plaques in Alzheimer's disease brain with a synthetic peptide. The Biotin-AECDWGKGGRWRLWPGASGKTEACGP peptide was used to stain brain sections from normal and AD brain. This peptide specifically stained amyloid plaques in AD brain. The peptide did not stain non-Aß amyloid in tissues from a patient with amyloidosis (kidney is shown). Bar is 100µm for panels on the left and 25 µm for panels on the right.

[0008] Figure 7 shows a model of how $Aß_{1-40}$ binding-peptides can be used. The cysteine-linked peptide sequences CDWGKGGRWRLWPGASGKTEAC (SEQ ID NO:5) and CPGRSPFTGKKLFNQEFSQDQC(SEQ ID NO:6) can be used to bind to amyloid plaques in Alzheimer's disease brain. These peptides could be used as carriers to deliver molecules to amyloid plaques that 1. lessen their neurotoxicity, 2. stimulate their destruction, or 3. inhibit their formation. In addition, such peptides could be conjugated to molecules used to 4. visualize amyloid plaques, or 5. induce an anti-ideotype antibody.

Detailed Description

[0009] Alzheimer's disease (AD) is the major cause of dementia in the elderly, affecting approximately 3-4 million people in the United States alone (Bachman et al.,

1992). The decline of cognitive abilities in AD is associated with pathologic changes in the brain, the most prevalent of which are the formation of amyloid plaques and neurofibrilary tangles(for review, see Selkoe, 2001). Amyloid plaques in AD brains form at far greater numbers than in normal individuals. While amyloid plaques contain many proteins, they have as their principal constituent the 4kDa amyloid-ß (Aß) peptide (Kang et al., 1987). The formation of the Aß peptide, and thereby Aß amyloid, arises from aberrant processing of the amyloid precursor protein (APP). A number of studies support the idea that Aß is itself neurotoxic (see Selkoe, 2001), and therefore the high concentration of Aß peptide in amyloid plaques may seed the generalized degeneration of neurons in surrounding areas (Morris and Price, 2001).

[0010] The logical approach to inhibiting AD would appear to be to inhibit the proteases, in particular the γ- and ß-secretase, that produce the Aß peptide. As individuals without AD also have plaques (Morris and Price, 2001), and as some Aß peptide is produced in people without AD (Koudinov et al., 1994), it is possible that such peptide processing may be a byproduct of necessary protease functions. As such, inhibiting APP processing may have unwanted and toxic consequences. Another approach would be

to design therapies that would either eliminate the toxic aspects of amyloid plaques or remove plaques from the brain altogether. For example, Aß toxicity is associated with the generation of reactive oxygen species (Christen, 2000; Parks et al., 200; Lovell et al., 2000) and with the accumulation of heavy metals (Cherny et al., 2001). Therefore, the creation of a reducing or chelating environment locally at amyloid plaques may inhibit the toxicity associated with Aß in these areas. Because many proteins in addition to Aß accumulate in amyloid plaques, the activation of proteases may also aid in plaque removal or lessen plaque number or plaque size. Blocking of the cellular receptors that mediate Aß toxicity in neurons may also have a therapeutic benefit.

[0011] All of these approaches would be greatly facilitated by the ability to target therapeutics directly to amyloid plaques. One way to do this would be to develop "carrier" reagents that specifically bind Aß amyloid and can be conjugated with therapeutic molecules. Such reagents would likely have to be specific for the amyloid form of the Aß peptide and not bind monomeric Aß sequence, as the Aß sequence, when uncleaved, is a natural part of the amyloid precursor protein (APP). One or more APP isoforms are expressed in almost every tissue of the body (Wasco et al.,

1993). These isoforms are essential for viability, as mice lacking more than one APP form die shortly after birth (Heber et al., 2000). Therefore, reagents that identify linear Aß sequence would likely bind APP and have unwanted consequences. In order to circumvent this problem, we have used a phage display approach to identify peptide sequences that can bind to Aß peptide when present in its amyloid form, but that do not bind to Aß as a monomeric peptide. Here we identify two such peptide sequences. These sequences may be useful in targeting therapies specifically to plaques in the brains of AD patients. In addition, they may provide new tools to image plaques in living patients so as to facilitate the diagnosis of Alzheimer's disease.

[0012] A phage peptide library encoding $5X10^7$ random 20 amino acid sequences was used to pan for binding to $Aß_{1-40}$ amyloid and against adherence to tissue culture plastic. We decided to use a library that would be cysteine cross-linked at its base so as to increase the chance that the inserted peptide would have some tertiary structure. In addition, even though phage libraries with larger peptide inserts contain fewer of the total number of possible peptide combinations, we surmised that longer sequences would be required to identify high affinity binding motifs for the 40 amino acid Aß peptide. After three rounds of

positive panning against the amyloid form of the $A\beta_{1-40}$ peptide, and two rounds of negative panning against tissue culture plastic, we sequenced ten individual clones and compared these to ten randomly picked sequences from the starting library (Table 1). We identified multiple clones of only two phage sequences that adhered to the $A\beta_{1-40}$ amyloid.

[0013] While the amplification of individual clones with the same sequence can be caused by factors unrelated to adhesion, such as superior phage growth or an advantage in replication, we thought that this had not occurred for four reasons. First, the identified sequences had 3-fold more bulky hydrophobic residues (phenylalanine or tryptophan) than did sequences randomly picked from the starting library. Phage expressing such sequences should, if anything, be at a disadvantage for growth. Second, when nonspecific factors select for particular phage clones in a library with such a large peptide insert, the enriched sequences typically have deletions, which did not occur in this case. Third, both of the peptide sequences isolated shared characteristics in common that were not typical in the starting library. Selected sequences shared bulky hydrophobic amino acids that were spaced at even intervals $[(W/F)X_5(W/F)X_{2/3}(W/F)]$ (SEQ ID NO:1), and had two positively

charged residues (and no negatively charged residues) in the X_5 region. Finally, because the insert size of this library is so large, the number of sequences represented ($5x10^7$) is only a small fraction of the total number of possible random sequences of 20 linear amino acids ($1x10^{26}$). Therefore, these may be the only peptides with this kind of bulky hydrophobic spacing present in the library.

[0014] To determine if the selected phage peptides would bind to amyloid $A\beta_{1-40}$, we used individual phage clones to stain $A\beta_{1-40}$ amyloid that had been immobilized on tissue culture plastic (Fig. 1). Phage clones expressing the DWGKGGRWRLWPGASGKTEA (SEQ ID NO:2) sequence stained $A\beta_{1-40}$ amyloid extremely well. By contrast, phage clones containing the PGRSPFTGKKLFNQEFSQDQ (SEQ ID NO:3) sequence stained $A\beta_{1-40}$ amyloid more poorly, but the level of staining was still significantly above background levels. No clones from the starting library stained any $A\beta_{1-40}$ aggregates when used at the same concentration, and no signal was observed in the absence of phage with secondary antibody alone. In addition, no phage clones stained monomeric peptide that had been immobilized on nitrocellulose (data not shown). The Aß aggregates identified by these peptides are larger than individual 2-12 nm Aß fibrils (Stine et al., 1996, Tucker et al., 2000) and more closely resemble large (1-

10μm) aggregates found in amyloid plaques (Roher et al., 1986) and in some preparations of $A\beta_{1-40}$ (Stine et al., 1996). These peptides may also identify individual Aß fibrils, however, such structures cannot be visualized by conventional light microscopy.

[0015] We next determined if phage clones would bind to monomeric $A\beta_{1-40}$ by performing Western blots on isolated $A\beta_{1-40}$ peptide (Fig. 2). No clones expressing peptides from either the starting or the final peptide sequences identified monomeric $A\beta_{1-40}$. Monoclonal antibodies to Aß peptide did bind, however, demonstrating proper transfer of the Aß peptide to nitrocellulose. The lack of staining or blotting of the isolated phage sequences to linear $A\beta_{1-40}$ peptide suggests that they specifically recognize the amyloid form of $A\beta_{1-40}$.

[0016] While phage panning can be a powerful approach to identifying novel peptide sequences, there is no guarantee that those sequences will maintain their structure when taken out of the context of the bacteriophage coat protein. To verify that the DWGKGGRWRLWPGASGKTEA peptide would identify $A\beta_{1-40}$ amyloid independently of its presence in bacteriophage, we produced this protein in recombinant form as a fusion protein with thioredoxin. We engineered cysteines at either end of the peptide in the fusion

construct, along with several other residues from the phage coat protein. The ultimate or penultimate residue was engineered to be a proline to mimic predicted beta turn at either side of the 20 amino acid insert. We added these sequences in order to maximize the chance that this peptide would maintain the structure it displayed when present in the bacteriophage coat protein. We called this protein Thio-Aß. Recombinant thioredoxin without the Aß-peptide binding sequence was called Thio.

[0017] After purification by nickel resin chromatography, we performed binding studies on Thio and Thio-Aß to immobilized $Aß_{1-40}$ amyloid (Fig. 3). Thio did not bind to $Aß_{1-40}$ amyloid at any concentration below 200µM. By contrast, Thio-Aß bound with high affinity to Aß1-40 amyloid. We measured a Kd of 60 nM and binding was saturating at 200 nM. To determine if Thio-Aß would bind to Aß amyloid that was present in the amyloid plaques found in Alzheimer's disease (AD) brains (Fig. 4), we next stained AD and normal brains with Thio and Thio-Aß. Thio-Aß bound highly and very specifically to amyloid plaques in AD brains, while it did not bind to normal brain samples. No staining of neurofibrillary tangles was evident (not shown). We intentionally allowed the background level of staining to rise to the point where cells could be seen in

these sections, however, amyloid plaques could be visualized long before this normally happened. Positive staining of amyloid plaques with Thio-Aß was seen in AD brains from multiple subjects and was absent in sections from several normal brain samples. Concentrations as low as 100 nM yielded positive staining for Thio-Aß. By contrast, Thio did not bind to either normal or AD brain at any of the concentrations used.

[0018] We next tested whether the 20 amino acid peptide would bind $Aß_{1-40}$ amyloid if synthesized chemically (Figure 5). To test the relative contribution of the flanking cysteines, we synthesized peptides that either had or did not have these residues. Both peptides were made with an N-terminal biotin label to allow identification using streptavidin. Biotin-DWGKGGRWRLWPGASGKTEA and Biotin-AECDWGKGGRWRLWPGASGKTEACGP were tested for binding to amyloid $Aß_{1-40}$. Biotin-AECDWGKGGRWRLWPGASGKTEACGP bound Aß1-40 amyloid with a Kd of 320 nM. By contrast, Biotin-DWGKGGRWRLWPGASGKTEA, which lacks flanking cysteines, did not bind significantly at any concentration below 5µM. This peptide, however, did show binding in the 10-80 µM range (not shown). Therefore, the terminal cysteines, which most likely form a disulfide bond, are needed to induce a

conformation of the 20 amino acid insert that allows high affinity binding to $A\beta_{1-40}$ amyloid.

[0019] We next repeated the staining of AD and non-AD brain using Biotin-AECDWGKGGRWRLWPGASGKTEACGP (Figure 6). We also stained several organs (kidney, brain, large bowel, small bowel, and prostate) from a non-AD patient with amyloidosis. As with Thio-Aß, we found that the synthetic peptide specifically stained amyloid plaques in AD brain. Again, we saw no staining of neurofibrillary tangles. As with binding to $A\beta_{1-40}$ amyloid *in vitro*, slightly higher concentrations were needed for staining of plaques relative to Thio-Aß. Concentrations as low as 500nM gave good staining with the peptide. Simultaneous staining of kidney sections containing non-Aß amyloid demonstrated that binding was specific for Aß amyloid. Thus, this peptide sequence is a high affinity probe for Aß amyloid both *in vitro* and *in vivo*, both within and outside the context of other recombinant protein sequences.

[0020] Using phage display, we have isolated two cysteine-linked 20 amino acid peptide sequences that bind to the amyloid form of $A\beta_{1-40}$. Neither of these sequences bind monomeric $A\beta_{1-40}$, and therefore these peptides specifically identify the amyloid form of the $A\beta_{1-40}$ protein. Both of these sequences share a $[(W/F)X_5(W/F)\ X_{2/3}(W/F)]$ structure in

common, and both have two positively charged (and no negatively charged) amino acids within the X_5 region. Given that phage libraries with inserts of this length only contain a small fraction of the total number of possible peptide sequences, there may be other sequences with this motif that bind with even higher affinity that could not be identified in the current experiments. Therefore, cysteine-linked peptides with this repeating hydrophobic motif may provide a scaffold for the design of other peptides that can bind to Aß amyloid with even higher affinity. Production and purification of one of these peptide sequences as a fusion protein with thioredoxin (Thio-Aß), or direct chemical synthesis of the peptide, created a high affinity binding protein for $Aß_{1-40}$ amyloid *in vitro*. These reagents also bound specifically to amyloid plaques in Alzheimer's disease (AD) brain. Therefore, at least one of the peptide sequences we have identified binds Aß amyloid independently of its expression in bacteriophage.

[0021] We envision five potential applications for such peptides (Figure 7). First, these peptides could be coupled to molecules designed to inhibit the toxicity of amyloid plaques. Peptides could be coupled to anti-oxidants to protect against oxidative damage caused by the Aß peptide or to chelators that could inhibit the accumulation of

toxic metals. Second, these peptides could be coupled to reagents that degrade plaques. Activators of tissue plasminogen, urokinase-type plasminogen, or matrix metalloproteases may stimulate the breakdown of amyloid plaque proteins (Tucker et al., 2000). While global activation of such proteases would likely be toxic, the coupling of protease activators to these peptides may increase their accumulation at plaques to such an extent that they could be used at non-toxic doses. Third, these peptides may be conjugated to reagents that inhibit plaque formation. Fourth, these peptides could be coupled to radionuclides or other markers to image amyloid plaques in living patients. Alzheimer's disease is currently diagnosed through cognitive measures on patient interview. These measures are time consuming, and post-mortem analysis of brains is currently required for a definitive diagnosis. Thus, at the moment, there is no way to measure to extent of brain pathology in living patients. Such a lack of a quantitative measure makes it difficult to diagnose the early stages of the disease and to make determinations as to the efficacy of various treatments. Finally, these sequences could be used to develop an anti-ideotype vaccine. Since these peptides bind Aß amyloid, they may mimic the Aß amyloid binding site of cellular receptors

involved in mediating the neurotoxic effects of Aß. If so, immunization using these peptides could stimulate the production of blocking antibodies to cellular binding sites for Aß amyloid.

[0022] The development of small peptides that bind to Aß may be superior for the diagnostic and treatment purposes to other molecules that are known to bind to Aß peptide. The molecules that can bind to Aß amyloid can be broken down into three groups: non-antibody proteins, antibodies, and small organic molecules. There are many proteins besides antibodies that have been reported to bind to Aß amyloid, including complement C1q A (Burdick et al., 1994), biglycan, decorin, versican (Snow et al., 1995), anti-chymotrypsin (Lukacs and Christianson, 1996), p75 NGF receptor (Yaar et al., 1997), perlecan (Snow et al., 1995; Castillo et al., 1997), alpha 2 macroglobulin (Hughes et al., 1998), apolipoprotein E (Yamauchi et al., 1999), transthyretin (Tsuzuki et al., 2000), laminin (Castillo et al., 2000), alpha 2 macroglobulin (Hughes et al., 1998), and the alpha 7 nicotinic acetylcholine receptor (Wang et al., 2000). Most of these proteins are large and would be unlikely to traverse the blood-brain barrier. In addition, many of these proteins are present in amyloid plaques in AD patients and may therefore contribute to neurodegeneration

in AD. A number of antibodies bind Aß peptide (eg. Balass et al., 1999; Koppel et al., 1996), but these too are unlikely to traverse the bloodbrain barrier at significant levels. Several groups of small organic molecules, many of which are dyes, bind to Aß peptide, including Congo red (Carter and Chou, 1998), thioflavins (Zhuang et al., 2001), napthalenes (Agdeppa et al., 2001), styrylbenzenes (Zhuang et al., 2001), and some antibiotics (Findeis, 2000). In addition, several inorganic heavy metals bind Aß peptide, including copper, zinc, and iron (Balakrishnan et al., 1998). Many of these reagents are likely to have toxic side effects if given to people.

[0023] For example, Congo red and thioflavins can, in modified forms, be DNA mutagens (Reid et al., 1983; Teicher et al., 1990). As the peptide sequences we have found are small and relatively hydrophobic, they may be able to traverse the blood-barrier more efficiently than many of the larger protein listed above, or at least be a more amenable to subsequent engineering in this regard. In addition, these peptides are less likely to have any significant toxicity when compared with small organic molecules and may in some instances be easier to conjugate with other reagents.

Materials

[0024] A cysteine-linked phage peptide library encoding $5X10^7$ random 20 amino acid insertions was a generous gift from David L. Jaye and Charles A. Parkos (Emory University). $A\beta_{1-40}$ peptide (DAEFKHDSGTEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV) was purchased from Bachem (Torrence, CA). Biotinylated anti-sheep M13 phage polyclonal antibody was purchased from 5 Prime -3 Prime (Boulder, CO). Alkaline phosphatase and horseradish peroxidase-coupled streptavidin were purchased from Boehringer Mannheim(Indianapolis, IN) and Jackson Immunochemicals (West Grove, PA). Radionucleotides for DNA sequencing were purchased from Amersham (Piscataway, NJ). Oligonucleotides were purchased from Genosys (The Woodlands, TX). Recombinant peptides fused to thioredoxin were made and purified using plasmids and reagents in the His-Patch ThioFusion expression system from Invitrogen (Carlsbad, CA). Anti-Aß monoclonal antibody (2066) was a generous gift from Edward Koo (UC San Diego). Synthetic peptides containing an N-terminal biotin were synthesized and purified by AnaSpec (San Jose, CA).

Phage panning and quantitation

[0025] Methods for phage panning and amplification were done much as previously described (Mazzuchcelli et al., 1999). $A\beta_{1-40}$ peptide was added at a concentration of 20 µg/ml in Tris-buffered saline (TBS pH 7.4) to wells of a 24 well tissue culture plate (Falcon - Becton Dickinson, Franklin Lakes, NJ). Numerous amyloid fibrils and aggregates were evident on the bottom of the plate after several days, as previously seen (Stine et al., 1996; Tucker et al., 2000), however, peptide was left on for five days to allow more complete aggregation of the peptide to its amyloid form. Round 1: Plates were washed with phage buffer (Hanks balanced salts with 1 mM $CaCl_2$ and 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4, and 0.5% BSA) 3 times for 5 minutes each. Both plates were then incubated with 15 µl (5-10 copies of every sequence) of starting library diluted into 300 µl of phage buffer for one hour with rocking on a shaker. Plates were washed 6 times for 5 minutes each with phage buffer, and bound phage were eluted with phage buffer containing 0.5% Tween 20. Eluted phage were incubated with starved K91 cells for 15-20 minutes at room temperature and grown in Luria Broth (LB) with 1 µg/ml kanamycin for 45 minutes on a bacterial shaker. A portion of this material was used to titer phage using a plaque assay as previously

described (Smith and Scott, 1993). Infected bacteria were then plated on 15cm LB-agar plates containing 75µg/ml kanamycin overnight at 37°C. Bacteria were scraped from plates and collected in 3 ml TBS per plate (pH 7.5). Bacterial suspension was transferred to Nalgene tubes and spun at 9,100g for 10 minutes. Supernatant was collected and phage precipitated in 0.15 volumes of PEG/NaCl (16.7% polyethylene glycol-8000/3.3M NaCl) on ice for two hours. Precipitated phage was spun at 9,100g at 4°C for 30 minutes and re-suspended in 150 mM NaCl, 10 mM HEPES pH 7.4. Phage were then re-precipitated as above and suspended in 150 mM NaCl, 10 mM HEPES pH 7.4. Rounds 2 and 3: Amplified phage from Round 1 was incubated on a tissue culture well without $A\beta_{1-40}$ peptide for one hour in phage buffer. Supernatant from this well was then incubated on a plate coated with amyloid Aß1-40 as in Round 1. All other procedures were done as described in Round 1.

DNA sequencing of phage clones

[0026] At the end of Round 3 of panning, phage-infected K91 cells were diluted and plated as single colonies on LB-Agar plates with 75µg/ml kanamycin. Individual colonies were grown up at 37 C for 12 hours in LB with 1 µg/ml kanamycin. Bacteria were spun down at 10,000g for five minutes, after

which the supernatant was precipitated in PEG/NaOAc (3.6%/450 mM) for 24 hours on ice. Precipitated phage DNA was isolated by spinning at 10,000g for 15 minutes and re-suspended in Tris-EDTA (TE, pH 7.5). Anti-phage primer was added (5'gtttgtcgtctttccagacg) and DNA sequencing reactions were run using the Sequenase sequencing kit (Amersham, Piscataway, NJ).

Staining and blotting with phage

[0027] 10^{11} PFU (plaque forming units)/ml of various phage clones were incubated with the amyloid form of $A\beta_{1-40}$ peptide. Aß amyloid was made by incubating peptide in TBS (pH 7.4) for 7 days on Falcon 24-well tissue culture wells. After washing, bound phage were visualized by incubation with biotinylated anti-M13 antibody and alkaline phosphatase-conjugated streptavidin, followed by incubation for equivalent times in 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT). No staining was seen with any phage on control plates lacking $A\beta_{1-40}$. Phage were also used to stain monomeric and amyloid forms of $A\beta_{1-40}$ peptide that had been immobilized on nitrocellulose (data not shown). The linear and amyloid forms of Aß were made as previously described (Tucker et al., 2000) and immobilized nitrocellulose-coated tissue

culture plates, also as previously described (Martin and Sanes, 1997). Phage clones selected to bind the amyloid form of Aß1-40 did not bind non-amyloid $Aß_{1-40}$ (data not shown). For immunoblotting, 10ng of monomeric $Aß_{1-40}$ was separated on a 4-12% Bis/Tris NuPAGE gradient gel (Novex; San Diego, CA). After transfer to nitrocellulose, blots were blocked in phage buffer and incubated with 10^{11} PFU/ml of each clone. After washing in phage buffer (without BSA), blots were incubated with biotinylated anti-M13 antibody followed by streptavidin-coupled horseradish peroxidase. Blots were developed using the ECL chemiluminescence method (Pierce, Madison WI). $Aß_{1-40}$ peptide was also blotted with a monoclonal antibody that recognizes the Aß peptide to confirm protein transfer.

Production of recombinant Aß-binding peptide

[0028] Complementary oligonucleotides encoding the DWGKGGRWRLWPGASGKTEA peptide sequence were annealed, digested with Kpn I and Xba I, and ligated into pThioHisC plasmid (Invitrogen; Carlsbad, CA) at the Kpn I and Xba I sites using the following sequences:

5'CGGGGTACCTGCAGAATGCGATTGGGGGAAGGGGGGTCGGTGGCGGTTGT
GGCCGGGTGCGTCGGGGAAGACGGAGGCGTGCGGCCCGCCGTATTAGTCTAG
AGC (forward) and

5'GCTCTAGACTAATACGGCGGGCCGCACGCCTCCGTCTTCCCCGACGCACCC
GGCCACAACCGCCACCGACCCCCCTTCCCCCAATCGCATTCTGCAGGTACCCC
G (reverse). This added several flanking amino acids from the phage coat sequence at either end of the 20 amino acid insert, such that the sequence around the insert site (beginning at the Kpn I site in pHisThioC) was PAEC-insert (DWGKGGRWRLWPGASGKTEA) -CGPPY-stop. XL1-Blue bacteria were transformed with plasmid either containing insert (pThio-Aß) or lacking insert (pThio). Transformed bacteria were grown and protein induced with 1mM IPTG in log phase. Cells were pelleted and harvested by repeated cycles of freezing and thawing with subsequent sonication. Recombinant Thio or Thio-Aß protein was purified by incubation with ProBond nickel chelating resin (Invitrogen; Carlsbad, CA). Protein was bound with pH 7.8 buffer, washed successively with pH 6.0 and pH 5.5 buffers, and eluted with pH 4.0 buffer according to the manufacturer's instructions. Eluted protein was immediately re-pHed to 7.5 after elution. Recombinant protein comprised ca. 80% of the protein in fractions used for binding studies.

Binding assays with recombinant Thio and Thio-Aß protein and synthetic peptides

[0029] $A\beta_{1-40}$ was immobilized on 96-well ELISA plates as described above for phage panning. Immobilized $A\beta_{1-40}$ was blocked in phage panning buffer (Hanks balanced salts with 1 mM $CaCl_2$ and 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4, and 0.5% BSA) for one hour. Thio or Thio-Aß protein was added at varying concentrations in phage panning buffer for 2 hours at room temperature. Plates were extensively washed in phage panning buffer without BSA. Anti-Thio antibody (Invitrogen; Carlsbad, CA) was added for 30 minutes in phage panning buffer at a dilution of 1:500. After washing, anti-mouse IgG conjugated to alkaline phosphatase was added at 1:500 for 40 minutes. Plates were washed again and incubated with paranitrophenyl phosphate (Sigma; St. Louis, MO). Developing substrate was read several times at 405 nm on an ELISA plate reader over the linear range (OD 0.2-1.0) and normalized to the highest binding signal. To calculate dissociation constants, binding curves were fitted by non-linear regression analysis assuming a single class of equivalent binding sites. Binding of primary and secondary antibody to $A\beta_{1-40}$ never exceeded 10% of the maximal signal for Thio-Aß in any experiment, and Thio protein never bound significantly above primary and secondary antibody alone at any concentration below 100µM. Thio did show some binding

in the mM range (data not shown). By contrast, Thio-Aß protein was saturating at 200nM.

[0030] For binding to synthetic peptides, two peptides were synthesized and purified containing an N-terminal biotin. One of these was the 20 amino acid insert without any flanking sequences, Biotin-DWGKGGRWRLWPGASGKTEA. The other sequence, Biotin-AECDWGKGGRWRLWPGASGKTEACGP, contained flanking cysteine residues and several other amino acids from the bacterial coat sequence. These peptides were purified by HPLC and confirmed by mass spectrometry to be over 90% pure. Peptides were solubilized in phage buffer and incubated at varying concentrations with $Aß_{1-40}$ amyloid for 1 hour. After washing in phage buffer as above, streptavidinconjugated to alkaline phosphatase was added at 1U/ml for 50 minutes. Plates or slides were washed extensively in phage buffer and developed, as above.

Staining of human brain with recombinant Thio-Aß-protein and synthetic peptides

[0031] Normal and AD brain samples were obtained from the UCSD Alzheimer's Research Center (La Jolla, CA). Sections from a non-AD amyloidosis patient were obtained from the Department of Pathology (UCSD). Paraffin-embedded samples

of cortex or other tissues were sectioned at 10μm and mounted on glass slides. After deparifinization, sections were fixed in formic acid, blocked in phage panning buffer, and incubated with 5-500nM Thio-Aß or Thio for two hours at room temperature. Binding was determined by subsequent staining with anti-Thio antibody and anti-mouse IgG coupled to alkaline phosphatase as above. After washing, staining was developed using 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium for identical periods of time. All washes and incubations were done in phage buffer. The existence of amyloid plaques was confirmed by binding of anti-Aß monoclonal antibody (2066) to sections from the same brain samples. No significant staining was ever observed with secondary antibody alone. Background staining was allowed to develop to the point where cells in the section were evident. Staining with Thio-Aß was confirmed in brains from multiple AD subjects and was negative in multiple non-AD controls.

[0032] For staining with synthetic peptide, tissue sections from AD and non-AD brain, as well as from brain, kidney, small bowel, large bowel, and prostate from a non-AD patient with amyloidosis, were cut and prepared as above. Biotinylated peptide (AECDWGKGGRWRLWPGASGKTEACGP) (SEQ ID NO:4) was added in phage buffer for 1 hour at

concentrations ranging from 0.1-10μM. Sections were washed with phage buffer, incubated with streptavidin coupled to alkaline phosphatase, washed and developed as above. Positive staining of peptide to plaques in AD brain (and negative staining of non-Aß amyloid) was confirmed by simultaneous staining of sections using the same reagents. Congo Red or hematoxylin and eosin staining was done to confirm the presence of amyloid in amyloidosis sections (not shown).

[0033] Table 1. Identification of peptides that adhere to $Aß_{1-40}$ amyloid. A random 20-amino acid cysteine-cross-linked phage peptide library with $5X10^7$ possible sequences was screened for adhesion to $Aß_{1-40}$ amyloid. Sequences of 10 randomly picked phage clones in the starting library are shown, as are sequences of 10 randomly picked phage clones isolated after three rounds of panning against $Aß_{1-40}$ amyloid. At least two peptides adhered to $Aß_{1-40}$ amyloid. These sequences shared a density of similarly spaced bulky hydrophobic amino acids (underlined) that were not present in clones picked from the starting library. Two positively charged amino acids (dark) were present between the first two hydrophobic residues in both peptides.

Random starting clone sequences:

1. LGSGRIGDGWSDGGLARRLK (SEQ ID NO:7)
2. DGGGGAGRWTTKDRSAAKTE (SEQ ID NO:8)
3. VDDGAQGKRWGGMGLGKGRR (SEQ ID NO:9)
4. SGSGVGLRMASQRHEGRKVY (SEQ ID NO:10)
5. QLPQNGGPAWFTRKAGQGGR (SEQ ID NO:11)
6. LGYAGGGQGMVEGSFWPTSW (SEQ ID NO:12)
7. GLRGMEGRGYPKDRRDRNLE (SEQ ID NO:13)
8. LIGGNKAGRGAWGVVASSGR (SEQ ID NO:14)
9. ELESRGGLGYAWRGSASTMD (SEQ ID NO:15)
10. KGETGNGGRAKAGTVDLIRR (SEQ ID NO:16)

Random final clone sequences:

1. DWGKGGRWRLWPGASGKTEA
2. DWGKGGRWRLWPGASGKTEA
3. DWGKGGRWRLWPGASGKTEA
4. DWGKGGRWRLWPGASGKTEA
5. DWGKGGRWRLWPGASGKTEA
6. PGRSPFTGKKLFNQEFSQDQ
7. DWGKGGRWRLWPGASGKTEA
8. PGRSPFTGKKLFNQEFSQDQ
9. DWGKGGRWRLWPGASGKTEA
10. DWGKGGRWRLWPGASGKTEA

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the globular domain repeats on the laminin a chain. *J. Neurosci. Res.* **62**, 451-462.

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What is claimed is

1. An isolated or recombinant polypeptide comprising the amino acid sequence:

[(Trp/Phe) Xaa_1 Xaa_2 Xaa_3 Xaa_4 Xaa_5 (Trp/Phe) Xaa_6 Xaa_7 (Trp/Phe)]

wherein X_1 is any amino acid,

X_2 is any amino acid,

X_3 is any amino acid,

X_4 is any amino acid,

X_5 is any amino acid,

X_6 is any amino acid,

X_7 is any amino acid,

and wherein at least two of the amino acid residues of X_1 through X_5 are positively charged.

2. An isolated or recombinant polypeptide comprising the amino acid sequence:

[(Trp/Phe) Xaa_1 Xaa_2 Xaa_3 Xaa_4 Xaa_5 (Trp/Phe) Xaa_6 Xaa_7 Xaa_8(Trp/Phe)]

wherein X_1 is any amino acid,

X_2 is any amino acid,

X_3 is any amino acid,

X_4 is any amino acid,

X_5 is any amino acid,

X_6 is any amino acid,

X_7 is any amino acid,

X_8 is any amino acid,

and wherein at least two of the amino acid residues of X_1 through X_5 are positively charged.

3. An isolated or recombinant polypeptide comprising the amino acid sequence set forth in SEQ ID NO:2.

4. An isolated or recombinant polypeptide comprising the amino acid sequence set forth in SEQ ID NO:3.

5. An isolated or recombinant polypeptide comprising the amino acid sequence set forth in SEQ ID NO:4.

6. An isolated or recombinant polypeptide comprising the amino acid sequence set forth in SEQ ID NO:5.

7. An isolated or recombinant polypeptide comprising the amino acid sequence set forth in SEQ ID NO:6.

8. The polypeptide of any one of claims 1-4, further including a disulphide bond.

9. The polypeptide of any one of claims 1-4, further including a cysteine residue at the N-terminus and a cysteine residue at the C-terminus.

10. The polypeptide of any one of claims 1 through 7, wherein the polypeptide binds to the amyloid form of the Aβ peptide.

11. The polypeptide of any one of claims 1 through 7, further comprising a therapeutic or diagnostic compound conjugated to the polypeptide.

12. The polypeptide of claims 1 or 2, wherein none of the amino acid residues of X_1 through X_5 are negatively charged.

13. A composition useful for treating or diagnosing Alzheimer's disease in a mammal comprising a pharmaceutically or diagnostically acceptable carrier and a therapeutically- or diagnostically-effective amount of a polypeptide as claimed in claim 11.

14. A method of treating or diagnosing Alzheimer's disease in a mammal in need of such treatment, which comprises

administering to the mammal a therapeutically- or diagnostically-effective amount of a polypeptide as claimed in claim 11.

15. An isolated nucleic acid sequence encoding the polypeptide of any one of claims 1 through 7.

16. A vector comprising the nucleic acid sequence of claim 15.

17. The vector of claim 16, wherein the vector is an expression vector.

18. A host cell comprising the vector of claim 17.

19. The host cell of claim 18, wherein the host cell is a eukaryotic cell.

20. A method of treating Alzheimer's Disease comprising conjugating a peptide with a therapeutic compound.

21. The method as in claim 20, wherein said peptide binds specifically to the amyloid form of the Ab1-40 peptide in plaques of Alzheimer's patients.

22. The method as in claim 20, wherein said peptide comprises 20 amino acids intramolecularly cross-linked by two cysteines.

23. A method of diagnosing Alzheimer's Disease comprising conjugating a peptide with a compound, said compound is used in imaging or quantitating the amyloid plaques in the cerebrospinal fluid or brains of normal and diseased patients.

Abstract

[0072] The accumulation of the Aß peptides in amyloid plaques correlates with pathologic changes that occur in the brains of patients with Alzheimer's disease (AD). The ability to directly target reagents to the amyloid form of the Aß peptide may allow the delivery of neuroprotective agents to make amyloid plaques less toxic, the delivery of amyloiddestroying molecules to eliminate plaques, or the delivery of reagents to prevent amyloid plaque formation. In addition, such reagents may be useful as diagnostic tools to quantitate the extent of amyloid plaque formation in AD patients. As a step toward these goals, we have used phage peptide display technology to identify peptides that bind specifically to the amyloid form of the $Aß_{1-40}$ peptide. Here we identify two 20 amino acid peptides with similar structural features that bind to the amyloid form of $Aß_{1-40}$ but not to monomeric $Aß_{1-40}$. A recombinant form of one of these peptides was produced in *E. coli* as a fusion protein with thioredoxin. After purification, this reagent bound $Aß_{1-40}$ amyloid *in vitro* with a Kd of 60 nM and specifically labeled amyloid plaques in AD brains. A chemically synthesized version of this peptide also bound $Aß_{1-40}$ amyloid and specifically stained amyloid plaques in AD brain. These

peptide sequences represent new potential carrier molecules to deliver medicines to amyloid plaques in AD patients and to image plaques in AD brains.

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REAGENTS THAT BIND THE AMYLOID FORM OF THE AB PEPTIDE

Paul Martin

15670-005P01

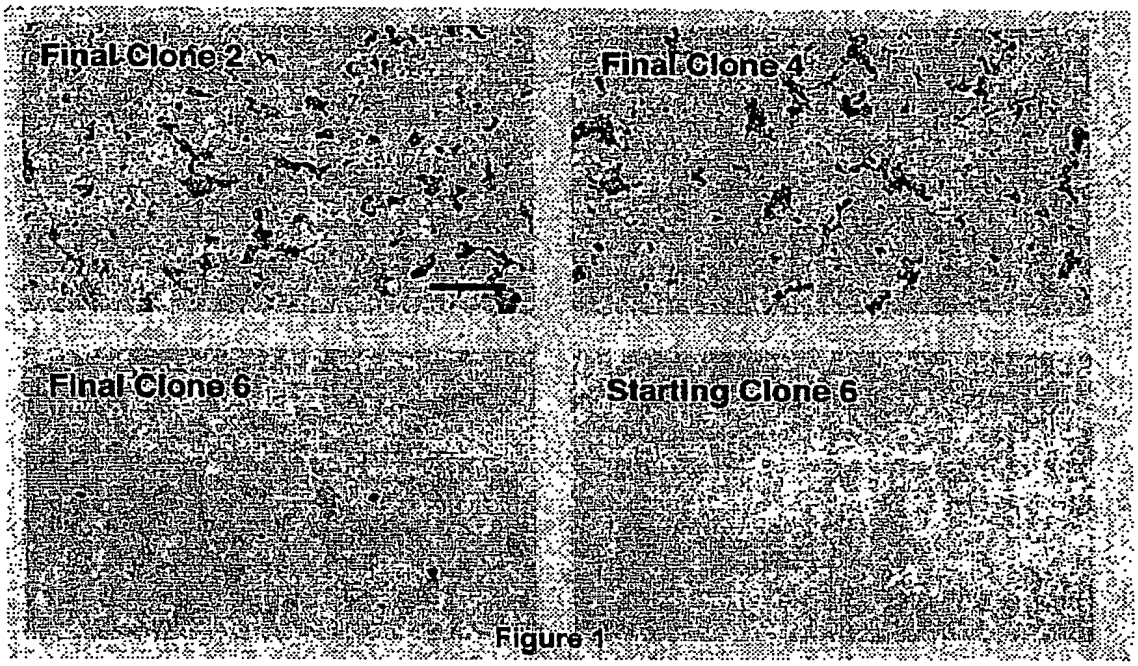
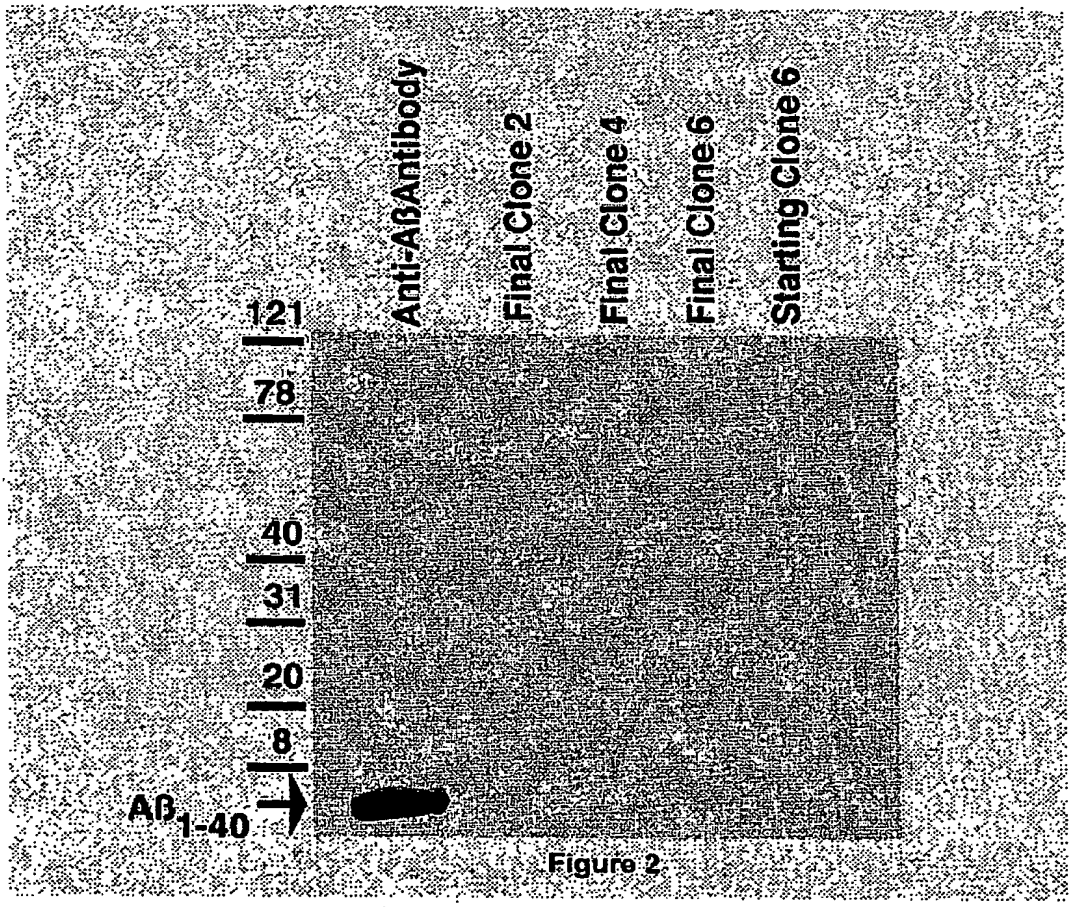


Figure 1


Anti-AβAntibody
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Final Clone 6
Starting Clone 6
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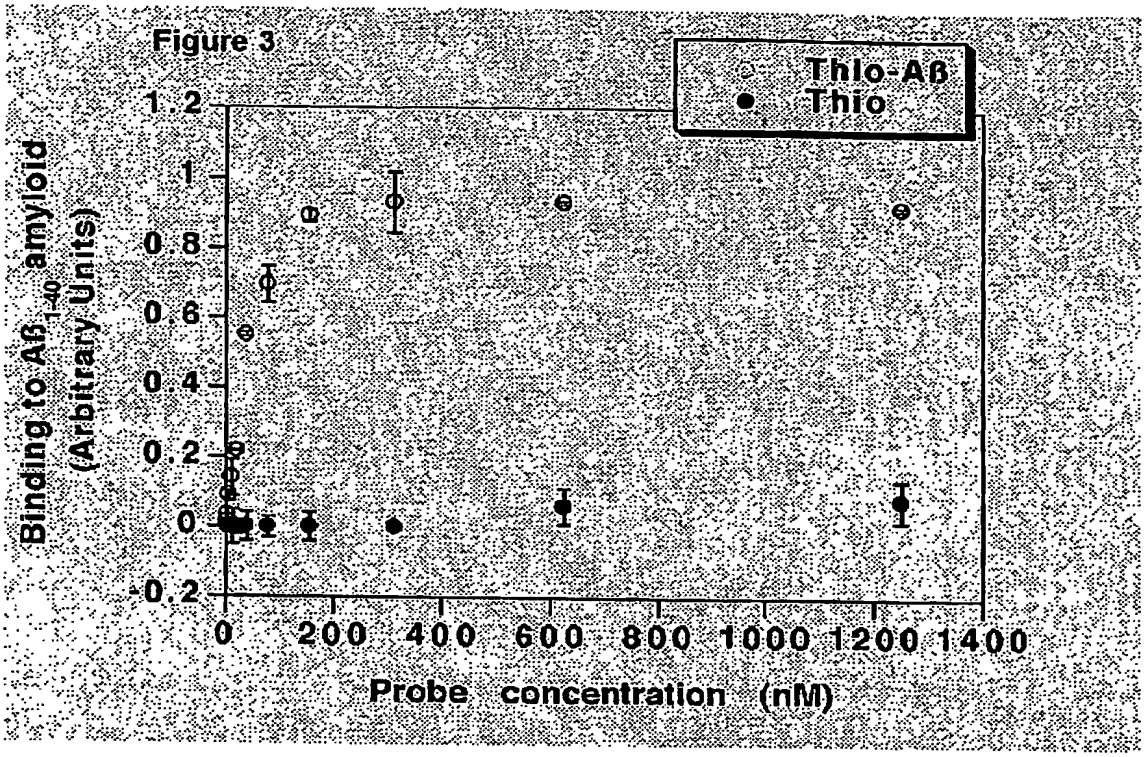
78
40
31
20
8
Aβ1-40

Figure 2



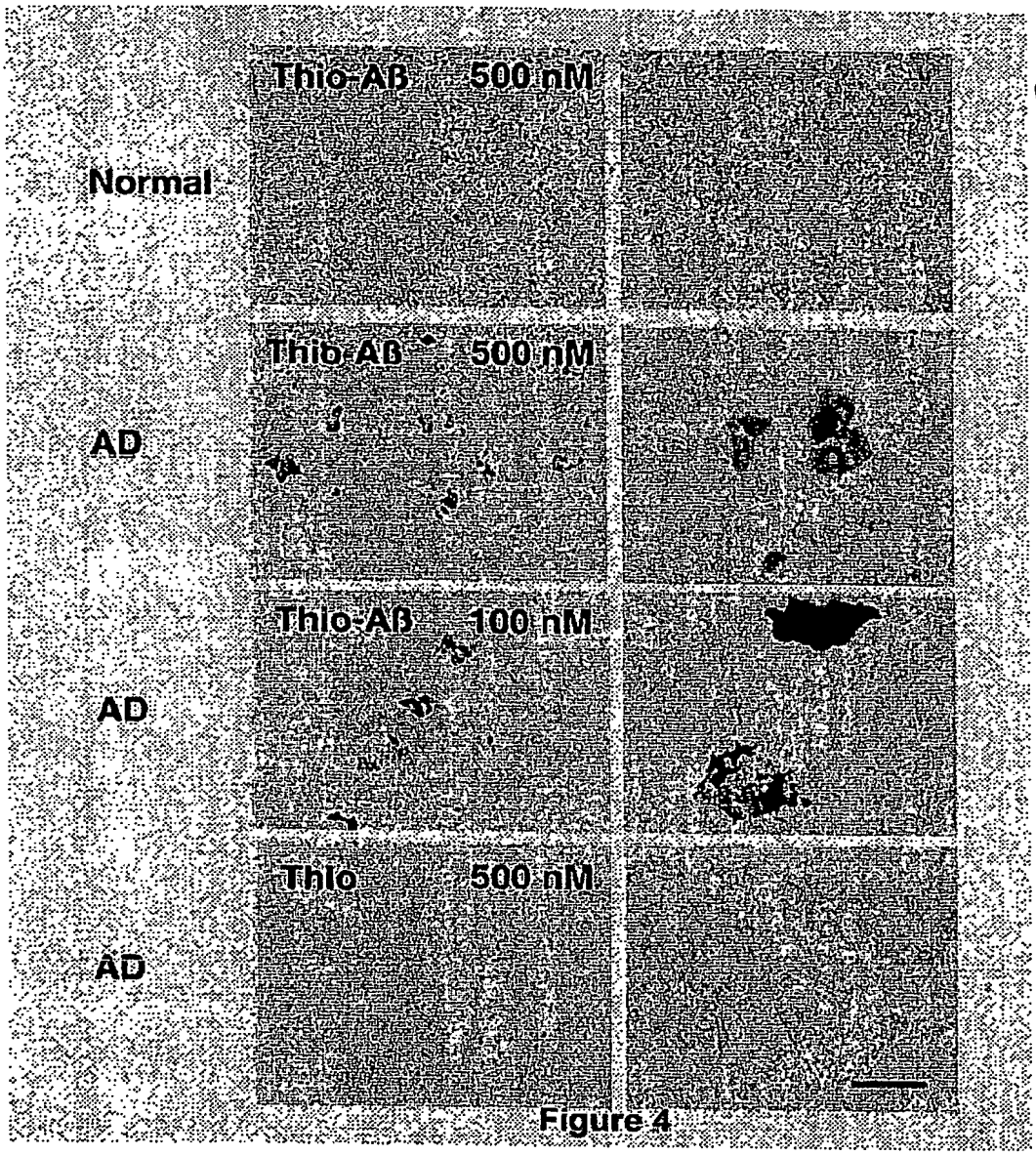


Figure 4

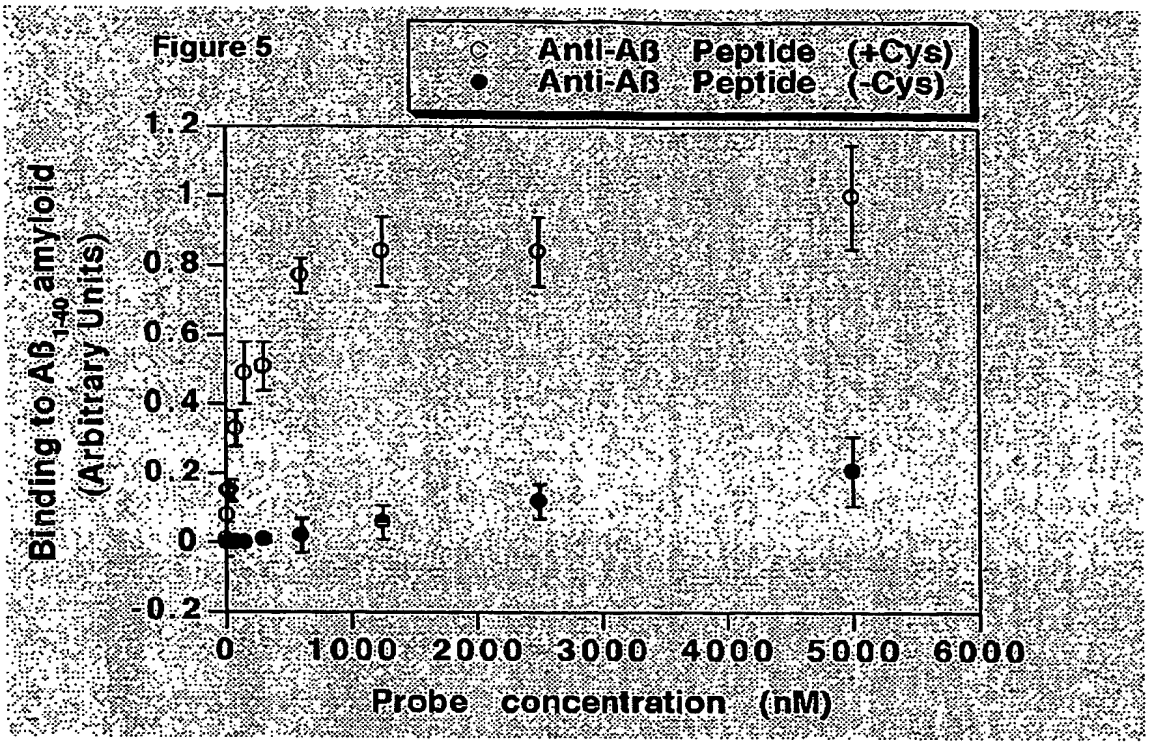


Figure 5
Anti-Aß Peptide (+Cys)
Anti-Aß Peptide (-Cys)
Binding to Aß1-40 amyloid (Arbitrary Units)
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
1000
2000
3000
4000
5000
6000
Probe concentration (nM)

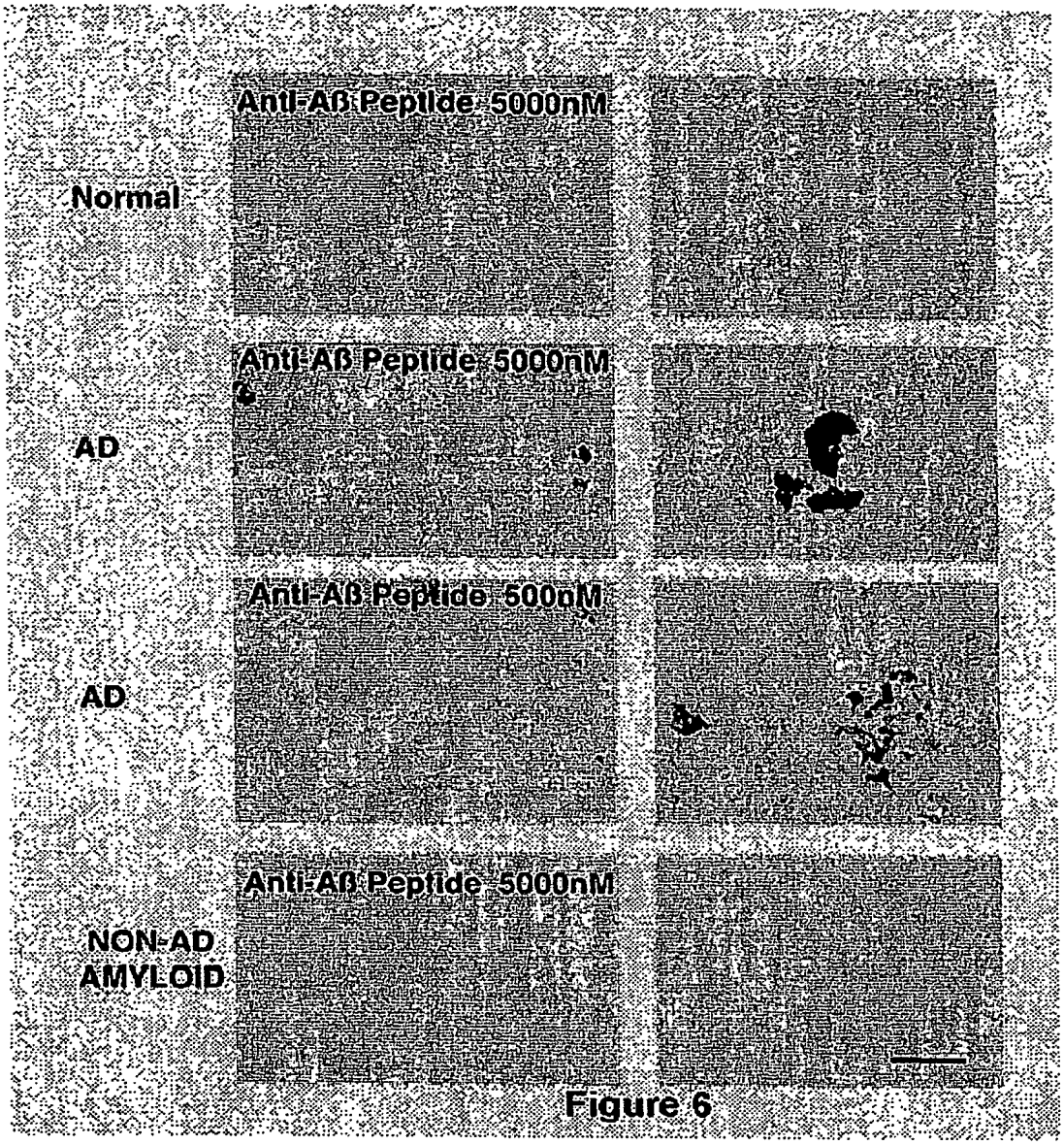


Figure 6

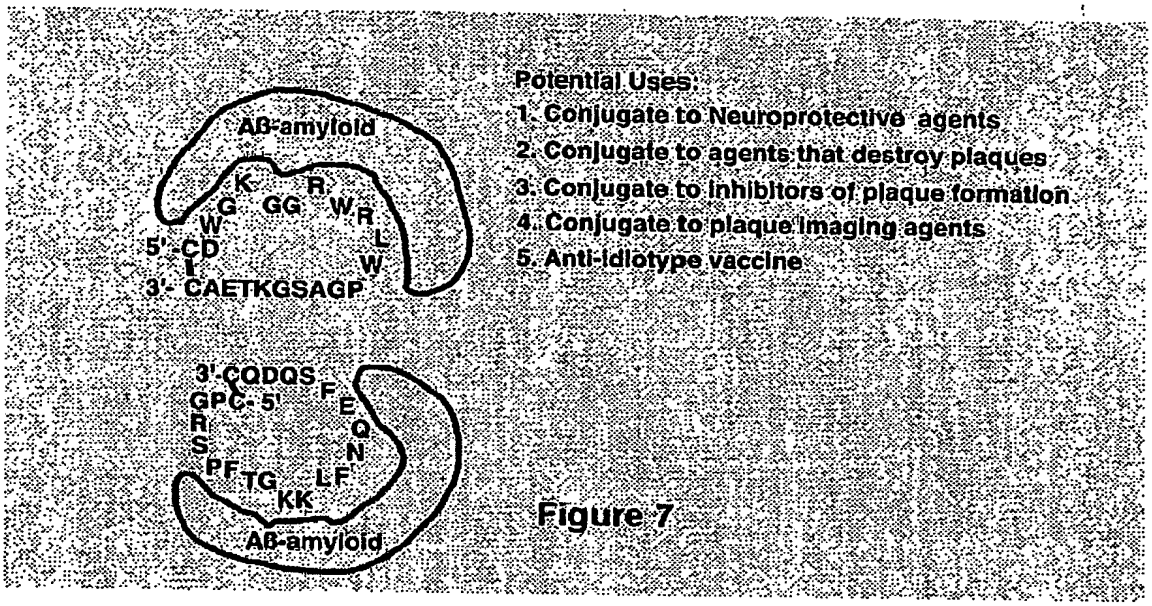

Aß-amyloid
K
G
GG
R
W
R
W
L
5'-CD
W
I
3'- CAETKGSAGP
3'-CODQS
GPC- 5'
F
E
R
Q
S
N
PF
TG
LF
KK
Aß-amyloid
Potential Uses:
1. Conjugate to Neuroprotective agents
2. Conjugate to agents that destroy plaques
3. Conjugate to inhibitors of plaque formation
4. Conjugate to plaque imaging agents
5. Anti-Idiotype vaccine

Figure 7

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